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Reduction in hepatic non-esterified fatty acid concentration after long-term treatment with atorvastatin lowers hepatic triglyceride synthesis and its secretion in sucrose-fed rats

Toshiyuki Funatsu [a,*], Masahide Goto [b], Hirotoshi Kakuta [a], Masanori Suzuki [a], Motoko Ida [b], Satomi Nishijima [b], Hideyuki Tanaka [a], Shuhei Yasuda [a], Keiji Miyata [a]

[a] *Pharmacology Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 3058585, Japan*

[b] *Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 3058585, Japan*

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Abstract

The mechanism by which atorvastatin lowers plasma triglyceride (TG) levels is mainly through a decrease in hepatic TG secretion. However, it is not clear why atorvastatin, which does not inhibit TG synthesis in vitro, decreases hepatic TG secretion without a prospective increase in hepatic TG concentration. For the investigation of the mechanisms that underlie the hypotriglyceridemic effects of atorvastatin, we characterized the effect of either a single or an 11 day administration of atorvastatin in sucrose-induced hypertriglyceridemic rats. Atorvastatin (30 mg/kg p.o.) strongly decreased the rate of both very-low-density lipoprotein (VLDL)-TG and VLDL-apolipoprotein B secretion. The inhibitor also decreased hepatic TG concentration. Hepatic TG synthesis activity was also decreased by atorvastatin, and its activity was correlated with both hepatic and plasma TG concentration. There was also a strong correlation between the hepatic TG synthesis and hepatic non-esterified fatty acid (NEFA) concentration ($r^2 = 0.815$). These effects required chronic administration of the inhibitor and were not observed by acute treatment. Repeated administration of atorvastatin also strongly reduced hepatic acyl-coenzyme A synthase mRNA levels. These results suggest that the reduced hepatic NEFA most likely lowers hepatic TG synthesis and TG secretion in sucrose-fed hypertriglyceridemic rats.

Keywords: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor; Fatty acid; Triglyceride synthesis; Atorvastatin; Acyl-coenzyme A synthase

1. Introduction

It is well established that hypercholesterolemia is a major risk factor for the development of coronary heart disease (CHD). Evidence based on animal experimental models and clinical studies shows that individuals with elevated concentrations of plasma low-density lipoprotein cholesterol (LDL-C) are at an increased risk of CHD [1 3]. Because of their ability to reduce elevated levels of LDL-C, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, have been used worldwide as first-line drugs for the management of hypercholesterolemia.

* Corresponding author. Fax: +81-298-54-1616.
E-mail address: funatsu@yamanouchi.co.jp (T. Funatsu).

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In addition to cholesterol dysfunction, increasing evidence suggests that elevated level of serum triglyceride (TG) might be an independent risk factor for CHD [4,5]. This has generated interest in the effect of statins on TG metabolism. Atorvastatin, a well-established member of the statin class, produces greater reductions in not only plasma LDL-cholesterol levels but also plasma TG than other statins [6,7]. This pronounced effect of atorvastatin is considered to be due to its long-lasting action, presumably a reflection of its longer half-life and the presence of active metabolites in the liver [8].

In vitro studies using the human hepatoma cell line, HepG2, demonstrated that atorvastatin reduced the number of hepatic apolipoprotein B (apoB)-containing lipoproteins. This occurred through a decrease in the concentration of cellular cholesteryl ester, which appears to be required for the assembly of nascent lipoproteins [9]. In vivo evidence using miniature pigs [10] and sucrose-fed rats [11] has shown that atorvastatin decreases the production rate of very-low-density lipoprotein (VLDL)-apoB and reduces the release of TG into plasma. This was accompanied by decreased secretion of apoB-containing lipoproteins. Since atorvastatin has no effect on TG synthesis within cells in vitro [12], the reduction in plasma levels of TG by atorvastatin is probably an indirect effect. Although inhibition of TG secretion from the liver without a reduction in TG synthesis may prospectively increase the TG content of the liver, no change in liver TG concentration was observed in these studies [10,11].

In order to understand why atorvastatin does not induce the compensatory increase in hepatic TG concentration, sucrose-fed rats, an animal model with endogenous hypertriglyceridemia, were treated with atorvastatin. Experiments were developed to investigate the effect of atorvastatin on hepatic TG synthesis, TG concentration and expression of enzymes involved in TG metabolism.

2. Materials and methods

2.1. Materials

Enzymatic lipid assay kits (cholesterol C-test, free cholesterol C-test, non-esterified fatty acid (NEFA) C-test and triglyceride G-test Wako) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The TaqMan probe was purchased from Genset K.K. (Tokyo, Japan). Isogen was from Nippon Gene (Toyama, Japan). [9,10(n)-^{3}H]Oleic acid (278 GBq/mmol) and [1-^{14}C]acetic acid (2.18 GBq/mmol) were obtained from Amersham Pharmacia Biotech (Tokyo, Japan). [^{3}H]Oleic acid was dissolved in 5 M NaOH, heated (60°C) for 15 min to give the sodium form and then diluted with saline [13]. Atorvastatin was a gift of Parke-Davis Pharmaceuticals Research (Ann Arbor, MI, USA). All other chemicals were of reagent grade.

2.2. Animals

Five- to 6-week-old male Sprague Dawley rats (SLC: SD) were purchased from SLC Japan (Hamamatsu, Japan). The animals were housed in metal cages in a temperature- (23 ± 2°C) and light cycle-controlled colony room (lights on 07.30 20.30 h) and had free access to water and standard rat chow (CE-2, Clea Japan, Tokyo, Japan). After matching for body weight, three groups of rats were used for these studies; one group was fed standard rat chow (normal group), while other groups were given a synthesized high sucrose diet during the experimental period (sucrose-induced hypertriglyceridemic groups). The sucrose-enriched diet (Oriental Yeast, Tokyo, Japan) contained 17% casein, 63% sucrose, 5% corn oil, 10% cellulose, 3.5% salt mixture, 1% vitamin mixture, and 0.5% choline chlorhydrate by weight, as described previously [14]. Hypertriglyceridemic rats were divided into two groups; rats in the control group received 0.5% carboxymethyl cellulose alone, while the atorvastatin group was given the compound (3 30 mg/kg body weight) suspended in 0.5% carboxymethyl cellulose by daily oral gavage for 11 days. In the case of single administration of atorvastatin, atorvastatin was given only on the final day following daily administration of the vehicle for 10 consecutive days. Since the high TG concentration of rats induced by sucrose diet has disappeared after an overnight fast [15], non-fasted rats were used in our study.

2.3. Determination of VLDL-triglyceride and -apoB secretion rate

Following treatment with atorvastatin, rats were anesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg), 1 h after the last administration of drug in the non-fasted state. Blood samples for evaluation of plasma VLDL were withdrawn from fundus oculi using capillary tubes (Funakosi, Tokyo, Japan). The in vivo rate of hepatic VLDL-TG secretion was examined following previously published methods [16]. Blood samples for the calculation of the rate of TG secretion were obtained from an abdominal vein 120 min after an intravenous injection of Triton WR-1339 (400 mg/kg; Sigma, St. Louis, MO, USA). Plasma was obtained by centrifugation at $1500\times g$ for 15 min at 4°C. VLDL (density (d) < 1.006 g/ml) was isolated at a density of 1.006 g/ml at $145\,000\times g$ at 16°C for 16 h after chylomicron isolation by centrifugation at $36\,000\times g$ at 16°C for 30 min. Plasma VLDL-TG concentration was determined by standard enzymatic procedures using commercially available kits. Plasma VLDL-apoB concentration was determined by the isopropanol method described previously [17]. The rate of TG secretion was calculated using the following formula: rate of TG secretion (mg/min) = $(TG_1-TG_0)/T\times$ $(0.0276\times BW+3.380)$, where T = 120 min, TG_0 is the TG concentration (mg/ml) before Triton injection, TG_1 is the TG concentration 120 min after Triton injection, and BW is the body weight of the animal (g). The rate of apoB secretion (μg/min) was calculated using this formula with apoB concentration instead of TG concentration.

2.4. Determination of hepatic lipid synthesis from [^{3}H]oleic acid

Sucrose-fed rats given atorvastatin were anesthetized with urethane (0.96 g/kg i.p.) 1 h after the last administration of drug. After blood samples for evaluation of plasma lipid levels were withdrawn from fundus oculi, [^{3}H]oleic acid was infused intravenously at 6 MBq/h/kg for 3 h. Following infusion, blood was rapidly withdrawn from the abdominal cava with a heparinized syringe, and a sample of liver was collected, frozen immediately with liquid nitrogen and stored at −40°C. Lipids in the liver homogenate and plasma were extracted in chloroform methanol (2:1), and the solvent evaporated using a centrifugal evaporator (ECD92D-2, Sakuma Seisakusho, Tokyo, Japan). TG, cholesteryl ester and phospholipid were separated on a silica gel G plate (Silicagel 60, Merck, Rahway, NJ, USA) using petroleum ether/diethyl ether/acetic acid (80:20:1) as a solvent system. These fractions were identified with iodine vapor, cut from the plate, and the radioactivity in the area corresponding to authentic standards counted in a liquid scintillation counter (2200CA, Packard, CT). To study the relationship between hepatic lipid concentrations and activity of TG synthesis from [^{3}H]oleic acid, hepatic lipid concentrations in the rats infused with [^{3}H]oleic acid were also determined.

2.5. Determination of hepatic cholesterol synthesis from [^{14}C]acetic acid

Sucrose-fed rats given atorvastatin for 11 days were used. One hour after the last administration of drug, rats received an intraperitoneal injection of [^{14}C]acetic acid sodium salt (7.4 MBq/kg). One hour later, the animals were anesthetized with diethyl ether and livers were excised, 0.5 g portions were weighed and saponified in 15% KOH/95% ethanol for 1.5 h at 75°C. Non-saponified lipids were extracted twice with n-hexane. Cholesterol was separated by the digitonin precipitate method described previously [18].

2.6. Determination of plasma and hepatic lipid concentrations

Samples of blood and liver obtained from rats that had received atorvastatin were used. Plasma was obtained by centrifugation at $1500\times g$ for 15 min at 4°C. Liver homogenates were extracted by the method of Folch et al. using chloroform methanol (2:1, v/v) as an extraction solvent [19]. Lipids were solubilized with a Triton X-100 solution, and hepatic TG, total cholesterol, free cholesterol and NEFA concentrations were determined enzymatically as described previously [20]. Cholesteryl ester mass was estimated by subtracting the free cholesterol mass from the total cholesterol mass. Plasma lipid concen-

trations were determined by standard enzymatic procedures using commercially available kits.

2.7. Determination of hepatic mRNA expression

Sucrose-fed rats received atorvastatin for 11 days and were anesthetized with pentobarbital (60 mg/kg i.p.) 1 h after the last administration of drug. A sample of liver was collected, frozen immediately with liquid nitrogen and stored at −80°C until use. Total RNA was isolated using 1 ml of Isogen according to the manufacturer's protocol. Using total RNA as a template, cDNA fragments were amplified with a SuperScript Preamplification System for First Strand cDNA Synthesis (Gibco BRL). Target mRNA content was determined using the ABI PRISM 7700 system (Perkin-Elmer Applied Biosystems, Alameda, CA, USA) [21], following RT-PCR which was carried out as described previously [22]. Oligonucleotide primers and TaqMan probes were designed using Primer Express, Version 1.0 (Perkin-Elmer Applied Biosystems) from the following sequences: long chain fatty acyl-CoA synthase (GenBank accession No. U15977), hepatic TG lipase (M16265), apoB (U53873), HMG-CoA reductase (NM013134), LDL receptor (X13722) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; X02231). The TaqMan probes, forward primers and reverse primers are shown in Table 1. TaqMan probes and primer pairs were purchased from Genset K.K. The quantitative differences between the cDNA samples were normalized by comparison with the GAPDH PCR products.

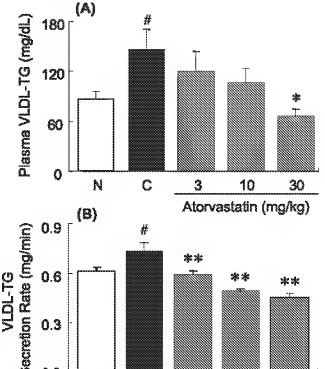

Fig. 1. Effect of atorvastatin on plasma VLDL-TG concentration and its secretion rate in sucrose-fed rats. Rats were maintained for 11 days on a normal rat chow diet (N, normal), sucrose-enriched diet alone (C, control) or with atorvastatin. (A) Plasma VLDL-TG concentration and (B) its secretion rate were determined as described in Section 2. Results are expressed as the mean ± S.E.M. of six to seven animals. $^{\#}P < 0.05$ vs. normal by Student's *t*-test, $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control by Dunnett's test.

2.8. Statistics

All results were analyzed using Statistical Analysis System ver. 6.11 (SAS Institute, NC, USA). Two-tailed Student's *t*-test was used for comparing two means, whereas the Dunnett multiple range test was used when three or more groups were compared. Results are presented as the mean ± S.E.M. Linear

Table 1
Sequences of the TaqMan probes, forward primers and reverse primers

Enzyme	TaqMan probe	Forward primer	Reverse primer
Acyl-CoA synthase	cgacgatgtcagaaccatgtacgat	tattcgaagatcagcagtcct	aggaccattatttgacacctg
Hepatic TG lipase	tccatgggtgggaagcgcaag	tttcaggattcgcaggca	agccctgtgattcttccga
ApoB	attccagcttcctcacctctcatgca	aggttcctgatcttcacataccag	gccgaaagcaggtatttcagtt
HMG-CoA reductase	caacgcccatgctgccaaca	ggctggtagcataggaggct	gcaatgtagatggcagtgacg
LDL receptor	agatgtgatgggcggaccgactg	cgatgcattcctgactcctg	ctagttcatccgagccatttttc
GAPDH	tgggctacactgaggaccagggttgtc	aagcaggcggccgag	atcaaaggtggaagaatggga

regression analysis was used to study the relationship between the variables.

3. Results

3.1. Plasma VLDL-TG and -apoB secretion rate

In animals fed sucrose, there was no difference in body weight gain or food consumption between control and statin-treated groups during the experimental period (data not shown).

The plasma VLDL-TG concentration in the sucrose control group rose 1.7-fold compared with that in the normal chow group (87 mg/dl versus 146 mg/dl; $P<0.05$, Fig. 1). Atorvastatin administration (3 30 mg/kg) for 11 days decreased plasma VLDL-TG concentration in a dose-dependent manner (Fig. 1). The higher dose (30 mg/kg) of the inhibitor resulted in a significant decrease by 55% ($P<0.05$). The rate of plasma VLDL-TG secretion was also increased by 19% in the sucrose-fed rats compared with the normal chow group ($P<0.05$, Fig. 1). Atorvastatin caused a concentration-dependent inhibition of the secretion rate of VLDL-TG, by 38% at 30 mg/kg ($P<0.01$).

Data on the VLDL-apoB concentration and its secretion rate for these animals are shown in Fig. 2. As seen with VLDL-TG, atorvastatin (30 mg/kg) lowered the VLDL-apoB concentration and the rate of its secretion by 56% ($P<0.05$) and 42% ($P<0.01$), respectively, whereas these parameters were slightly higher for the sucrose diet group than for the normal diet group, but no significant difference was noted. On the other hand, the changes in VLDL-apoB secretion by atorvastatin administration were of similar magnitude to those of VLDL-TG.

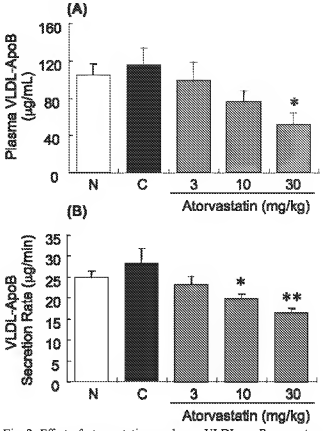

Fig. 2. Effect of atorvastatin on plasma VLDL-apoB concentration and secretion rate in sucrose-fed rats. Rats were maintained for 11 days on a normal rat chow diet (N, normal), sucrose-enriched diet alone (C, control) or with atorvastatin. (A) Plasma VLDL-apoB concentration and (B) its secretion rate were determined as described in Section 2. Results are expressed as the mean ± S.E.M. of six to seven animals. $*P<0.05$ and $**P<0.01$ vs. control by Dunnett's test.

3.2. Hepatic lipid concentrations

To determine whether the reduction in hepatic VLDL-TG secretion was accompanied by a compensatory increase of TG concentration in the liver, the

Table 2
Effect of atorvastatin on hepatic lipid concentrations in sucrose-fed rats

	n	Hepatic lipid concentrations (mg/g tissue)		
		FC	CE	TG
Control	7	2.1 ± 0.03	1.4 ± 0.09	11.4 ± 0.80
Atorvastatin 30 mg/kg	6	2.2 ± 0.04 (+4%)	1.1 ± 0.07* (−21%)	7.7 ± 0.63** (−33%)

Rats were maintained for 11 days on a sucrose-enriched diet alone (control) or with atorvastatin 30 mg/kg p.o. Hepatic lipid concentrations were determined as described in Section 2. Results are expressed as the mean ± S.E.M. of six to seven animals. $*P<0.05$ and $**P<0.01$ vs. control by Student's t-test. FC, free cholesterol; CE, cholesteryl ester; TG, triglyceride.

hepatic lipid concentration in atorvastatin-treated rats was measured (Table 2). Atorvastatin treatment (30 mg/kg) resulted in a significant decrease in hepatic TG concentration by 33% ($P < 0.01$) after daily doses for 11 consecutive days. In addition, the cholesteryl ester concentration was also decreased significantly by 21% ($P < 0.01$). These reductions were not seen when atorvastatin was administrated only on the last day of the sucrose-fed period (data not shown).

3.3. Hepatic lipid synthesis

To test the possibility that the decrease in hepatic TG concentration seen only after repeated administration of atorvastatin was due to an effect on hepatic TG synthesis, incorporation of [^{3}H]oleic acid into the hepatic TG fraction (TG synthesis activity) was also examined in the sucrose-fed rats. Preliminary experiments demonstrated that most of the infused exogenous [^{3}H]oleic acid was incorporated into the TG fraction, and that very low levels of radioactivity (2–4% compared with the TG fraction) were incorporated into cholesteryl ester and phospholipid fractions. Nevertheless, the radioactivity of these lipid fractions was linearly related to the concentration of infused [^{3}H]oleic acid within the range of 1.5–6 nEq. The total mass of infused oleic acid (6 nEq) was less than 1/3000 of the total plasma fatty acid content in a body (around 20 μEq), and was therefore unlikely to have a substantial effect on the results.

A single administration of atorvastatin on the final day did not affect hepatic lipid synthesis from infused [^{3}H]oleic acid (Fig. 3). In contrast, after treatment with atorvastatin for 11 days, the incorporation of [^{3}H]oleic acid into the hepatic TG fraction was significantly reduced by 29% ($P < 0.01$) compared to incorporation without an inhibitor (Fig. 3). The incorporation of radioactivity into the phospholipid fraction tended to be higher in the atorvastatin group than in the control group, although no significant difference was found ($P = 0.14$). Cholesteryl ester synthesis from oleic acid was not affected by treatment, while cholesterol synthesis from acetic acid was strongly inhibited by 69% ($P < 0.01$) compared to the control group (data not shown). There was a high correlation coefficient between the hepatic TG

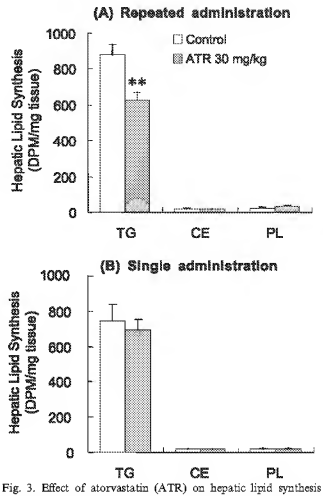

Fig. 3. Effect of atorvastatin (ATR) on hepatic lipid synthesis from exogenous [^{3}H]oleic acid substrate in sucrose-fed rats. Rats were maintained for 11 days on a sucrose-enriched diet, and given vehicle (control) or atorvastatin 30 mg/kg p.o. either during (A) the experimental period (repeated administration), or (B) only on the final day (single administration). The hepatic lipid synthesis was determined as described in Section 2. Results are expressed as the mean ± S.E.M. of six to seven animals. $^{**}P < 0.01$ vs. control by Student's *t*-test. TG, triglyceride; CE, cholesteryl ester; PL, phospholipid.

synthesis activity and hepatic TG concentration ($r^2 = 0.752$, $P < 0.01$, Fig. 4). There was also a significant correlation between the hepatic TG synthesis activity and plasma TG concentration ($r^2 = 0.501$, $P < 0.01$). In contrast, no relationship was found between the hepatic cholesteryl ester synthesis and plasma TG concentration ($r^2 = 0.017$, data not shown).

3.4. mRNA levels related to TG metabolism

To determine whether the change in hepatic TG

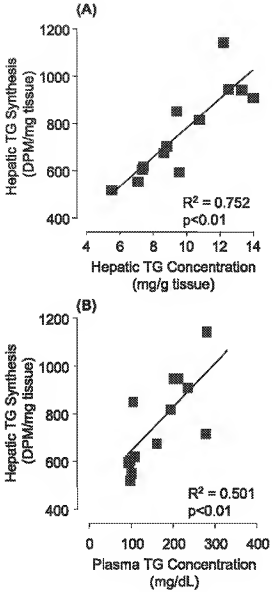

Fig. 4. Correlation between hepatic TG synthesis and (A) hepatic or (B) plasma TG concentration. (A) Amount of incorporation of [^{3}H]oleic acid into hepatic TG (TG synthesis) versus hepatic TG concentration in sucrose-fed rats ($r^2 = 0.752$, $P < 0.01$). (B) Amount of incorporation of [^{3}H]oleic acid into hepatic TG versus plasma TG concentration in sucrose-fed rats ($r^2 = 0.501$, $P < 0.01$). TG, triglyceride.

synthesis was related to the expression of lipogenic and lipolytic enzymes, mRNA levels of acyl-CoA synthase and hepatic TG lipase were measured. The effects of atorvastatin on mRNA levels of hepatic enzymes in sucrose-fed rates are given in Table 3. Repeated administration of atorvastatin reduced

Table 3
Effect of atorvastatin on mRNA levels of hepatic enzymes in sucrose-fed rats

Hepatic enzymes	Relative mRNA levels	
	Control	Atorvastatin 30 mg/kg
Acyl-CoA synthase	1.00 ± 0.15	0.10 ± 0.04**
Hepatic TG lipase	1.00 ± 0.15	0.73 ± 0.13
ApoB	1.00 ± 0.06	0.86 ± 0.08
HMG-CoA reductase	1.00 ± 0.05	1.37 ± 0.27
LDL receptor	1.00 ± 0.01	1.24 ± 0.29

Rats were maintained for 11 days on a sucrose-enriched diet alone (control) or with atorvastatin 30 mg/kg p.o. The relative mRNA levels were normalized to each control mRNA level as described in Section 2. Results are expressed as the mean ± S.E.M. of six to eight animals. **$P < 0.01$ vs. control by Student's t-test.

acyl-CoA synthase expression by around 90% ($P < 0.01$). mRNA expression of hepatic TG lipase, apoB, HMG-CoA reductase and LDL receptor was not significantly affected by atorvastatin administration.

3.5. Relationship between hepatic NEFA concentration and TG synthesis

As the statin-mediated down-regulation of the acyl-CoA synthase was considered to reflect the reduction of hepatic NEFA concentration, it was expected that hepatic NEFA concentration would be lowered by atorvastatin treatment. As excepted, repeated administration of atorvastatin reduced hepatic NEFA concentration by 19% ($P < 0.01$, Fig. 5). The correlation coefficient (r^2) between the hepatic NEFA concentration and hepatic TG synthesis activity was 0.815 ($P < 0.01$, Fig. 6). The reduction of hepatic NEFA concentration was not seen after a single administration of atorvastatin (Fig. 5).

4. Discussion

The main objective of the present study was to address the TG lowering mechanisms of atorvastatin in sucrose-fed rat, an animal model that has been shown to simulate endogenous hypertriglyceridemia associated with hyperinsulinemia [23] and increased VLDL-TG secretion [24].

Both VLDL-TG concentration and the rate of its secretion were increased by the sucrose diet by 67% and 19%, respectively (Fig. 1). Since the rate of VLDL-apoB secretion was slightly higher but no significant difference was noted, this indicates that a sucrose diet produces not only an increasing number of VLDL particles secreted but also larger VLDL particles rich in TG.

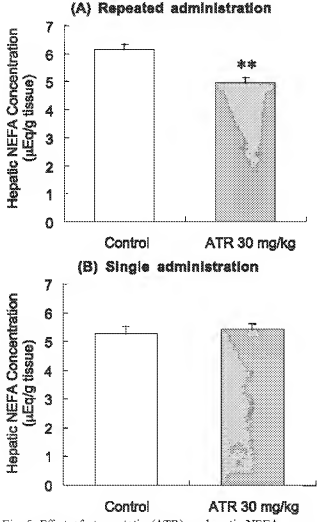

Fig. 5. Effect of atorvastatin (ATR) on hepatic NEFA concentration in sucrose-fed rats. Rats were maintained for 11 days on a sucrose-enriched diet, and given vehicle (control) or atorvastatin 30 mg/kg p.o. either during (A) the experimental period (repeated administration), or (B) only on the final day (single administration). Hepatic lipid concentrations were determined as described in Section 2. Results are expressed as the mean ± S.E.M. of six to seven animals. $^{**}P < 0.01$ vs. control by Student's *t*-test. NEFA, non-esterified fatty acid.

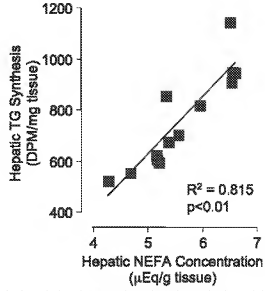

Fig. 6. Correlation between hepatic TG synthesis and hepatic NEFA concentration. The data from Figs. 3 and 5 were integrated. Amount of incorporation of [^{3}H]oleic acid into hepatic TG (TG synthesis) versus hepatic NEFA concentration in sucrose-fed rats ($r^2 = 0.815$, $P < 0.01$). NEFA, non-esterified fatty acid; TG, triglyceride.

Atorvastatin strongly decreased both plasma VLDL-TG concentration and the VLDL-TG secretion rate in sucrose-fed rats (Fig. 1). Since the extent of inhibition was almost comparable with that on the VLDL-apoB secretion rate, the secreted VLDL number of molecules was reduced by atorvastatin treatment without influence on its component. These results agree with the decrease in VLDL-TG and VLDL-apoB in other studies including human and animal models [10,25,26].

On the other hand, it is possible that inhibition of TG secretion from the liver may induce a prospective increase in liver TG mass. Indeed, subjects with abetalipoproteinemia develop hepatomegaly with fat-filled hepatic cells as a secondary consequence of the inability of these cells to incorporate TG into lipoprotein particles for secretion [27]. It has also been reported that statins slightly increase the cellular TG concentration in in vitro models using HepG2 cells and hamster hepatocytes [28,29]. It appears that the increase in the hepatocyte TG concentration is limited to in vitro models, since it has been reported that statins inhibited hepatic TG secretion but had no effect on hepatic TG concentration in several in vivo models [10,11].

Interestingly, our experiments indicated that repeated administration of atorvastatin caused not only inhibition of hepatic TG secretion but also a reduction in hepatic TG concentrations (Table 2). Furthermore, atorvastatin caused a 29% decrease in the incorporation level of [^{3}H]oleic acid into hepatic TG, namely TG synthesis (Fig. 3). The hepatic TG synthesis activity correlated significantly with both the hepatic TG concentration ($r^2 = 0.752$, $P < 0.01$) and plasma TG concentration ($r^2 = 0.501$, $P < 0.01$), indicating a possible causal relationship between these parameters (Fig. 4). In contrast, no relationship was found between the hepatic CE synthesis and plasma TG concentration. These findings suggest that the reduction in TG synthesis decreases hepatic TG concentration, which in turn decreases plasma TG concentration. However, a single administration of atorvastatin which inhibited de novo cholesterol synthesis almost completely in our previous result [30] did not affect either TG synthesis or hepatic TG concentration. These data indicate that inhibition of TG synthesis is considered not to be based on a direct effect of atorvastatin on de novo TG synthesis in the liver, similar to our previous in vitro study [12].

In order to understand how atorvastatin inhibits hepatic TG synthesis, we investigated changes in mRNA expression of enzymes related to TG metabolism during treatment. As shown in Table 3, atorvastatin induced a dramatic reduction (90%) in the mRNA level of acyl-CoA synthase in the liver. For the biosynthesis of TG in hepatocytes, fatty acids need to be activated because of thermodynamic considerations [31]. This reaction is catalyzed by acyl-CoA synthase. Since acyl-CoA synthase activity varies as a function of substrate availability [31], the fall in mRNA level of acyl-CoA synthase is considered to have resulted from the down-regulation which was caused by the decreased supply of hepatic fatty acids. Indeed, atorvastatin administration was associated with reduced hepatic NEFA concentrations (Fig. 5). Based on these results, decreased availability of hepatic NEFA may be responsible for the reduction in acyl-CoA synthase expression leading to lowered hepatic TG synthesis activity.

Why atorvastatin decreases hepatic NEFA concentrations is not completely understood. Although there have been few reports on the effect of statins on fatty acid metabolism, one study demonstrated a marked increase of fatty acid oxidation in rat liver when the lovastatin was administered for 1 week, which was accompanied by an activation of carnitine palmitoyltransferase I [32]. Similar to our results of hepatic NEFA reduction, acute treatment of the inhibitor did not have this activation. It has also been reported that statins stimulate peroxisome proliferator-activated receptor α (PPARα) activity by inhibiting the mevalonate pathway, which is considered to activate fatty acid β-oxidation reactions [33,34]. From these results, one could argue that the decrease in hepatic NEFA concentration following atorvastatin treatment may be due to enhanced activity of hepatic PPARα and/or mitochondrial carnitine palmitoyltransferase I caused by statins' chronic inhibitory activity of cholesterol synthesis. Although the decrease in plasma TG levels should be an effect common to all statins, atorvastatin may be more effective as a result of its longer half-life compared to other statins [35].

In summary, the results of our study further support the clinical evidence that the reduction in plasma VLDL-TG concentration provided by atorvastatin is a result of the reduction in secretion of hepatic TG-containing lipoprotein. We have also shown that repeated, as opposed to single, administration of atorvastatin reduced hepatic TG synthesis, and that this reduction was correlated with plasma TG concentration, indicating that the reduction in hepatic TG synthesis would lead to decreased plasma TG concentration. We have also shown that hepatic TG synthesis activity was highly correlated with hepatic fatty acid concentration. Hepatic acyl-CoA synthase expression was also reduced. Taken together, the data suggest that the reduced hepatic NEFA caused by long-term treatment with atorvastatin most likely lowers hepatic TG synthesis and TG secretion.

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